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Conjugative Transferability of the A/C Plasmids from *Salmonella enterica* Isolates That Possess or Lack bla_{CMY} in the A/C Plasmid Backbone

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Abstract

The objective of this study was to understand the conjugative transmissibility of resistance plasmids present in 205 *Salmonella enterica* isolates from bovine sources. Polymerase chain reaction (PCR)–based replicon typing was used to type plasmid replicons. Conjugation experiments were preformed in triplicate at 30°C and 37°C on solid medium. PCR mapping of the A/C transfer gene operon was done on 17 *Salmonella* Newport isolates that were only positive for A/C. Eighty-six percent ($n = 177$) of the *Salmonella* isolates were multidrug resistant (MDR) with resistance to 3–12 antimicrobial agents. Of these, 82% ($n = 146$) were resistant to extended-spectrum cephalosporins and possessed a bla_{CMY} gene. A/C was the predominant replicon detected, present in 90% ($n = 160$) of the MDR isolates. Twenty-three percent ($n = 37$) of the A/C-positive strains were positive for a second replicon. Replicons coresident with A/C included I1, N, B/O, HI1, and HI2. Only 31% ($n = 54$) of the MDR isolates produced transconjugants, and most of these donors carried multiple replicons. A/C cotransferred with B/O, N, and I1 at both 30°C and 37°C and with HI2 at 30°C. Seven *Salmonella* Newport isolates that produced transconjugants possessed only the single A/C replicon and lacked bla_{CMY}. PCR mapping of the A/C transfer gene operon in ten *Salmonella* Newport isolates that carried bla_{CMY} revealed a bla_{CMY} inverted repeat element integrated between the *traA* and *traC* genes. These results suggest that A/C may have been a conjugative plasmid before the integration of bla_{CMY} into the transfer gene operon. Additionally, transfer deficient A/C replicons may be mobilized in the presence of certain compatible conjugative plasmids.

Introduction

The emergence of multidrug-resistant (MDR) foodborne pathogens such as *Salmonella* and *Escherichia coli* has become an issue of increasing concern for both the food safety and medical communities. In 2002 a multistate outbreak of *Salmonella enterica* serotype Newport occurred in five states in the northeastern United States (CDC, 2002). This outbreak was associated with the emergence of MDR *Salmonella* Newport that is resistant to nine antimicrobial agents, including extended-spectrum cephalosporins (CDC, 2002; Gupta *et al.*, 2003). Cephalosporin resistance in *Salmonella* Newport has been linked to the AmpC-like β-lactamase gene bla_{CMY-2} and has been defined as *Salmonella* Newport MDR-AmpC (Winokur *et al.*, 2001; Carattoli *et al.*, 2002; Giles *et al.*, 2004). *Salmonella* Newport MDR-AmpC is recognized as an epidemic strain in humans and animals in the United States (CDC, 2002; Gupta *et al.*, 2003; Zhao *et al.*, 2003; Alcaine *et al.*, 2005; Cobbold *et al.*, 2006).

Localization of antimicrobial resistance genes on mobile genetic elements such as broad-host range plasmids, transposons, and integrons facilitates the horizontal transfer of these genes among bacteria and provides a rapid means of dissemination at the molecular level. Horizontal transfer of plasmids is mediated by a family of conjugation proteins, including transfer proteins, encoded by *tra* genes, that are a subset of bacterial type IV secretion systems (Christie *et al.*, 2005). Transfer of DNA is mediated by a nucleoprotein particle composed of a protein component that is covalently bound to the DNA (Christie *et al.*, 2005).

Plasmids have the capacity to replicate in many different bacterial genera; however, no two plasmids with the

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same replication control elements can replicate in the same bacterial cell. This characteristic is used to classify plasmids into incompatibility (Inc) groups, for example, IncA/C or IncN (Couturier *et al.*, 1988). However, multiple unrelated replication genes can exist on one plasmid, thus complicating incompatibility classification (Couturier *et al.*, 1988). For this reason, replicon typing based on the hybridization of individual replicons was developed; however, this process was lengthy and labor intensive (Couturier *et al.*, 1988). A recently described polymerase chain reaction (PCR)–based replicon typing (PBRT) method has greatly simplified typing of plasmids carried by Enterobacteriaceae (Carattoli *et al.*, 2005). For a review of incompatibility plasmids see Carattoli (2009).

Although characterization of resistance traits carried by plasmids has been extensive, identification of transmissible plasmid replicons is just beginning to shed light on the molecular epidemiology of these plasmids among foodborne pathogens (Johnson *et al.*, 2007). In many cases, researchers have found it difficult to transfer these large plasmids by conjugation *in vitro* and have resorted to electroporation to incorporate purified plasmids into recipient bacterial cells (Winokur *et al.*, 2000). One IncA/C plasmid, p254, isolated from a *Salmonella* Newport MDR-AmpC has been sequenced (Welch *et al.*, 2007). An IncA/C variant defined as A/C_2 (Carattoli *et al.*, 2006) has recently been described among six *Salmonella* and four *E. coli* plasmid donors submitted to the U.S. National Antimicrobial Monitoring System (NARMS) (GenBank accession no. AM087198).

The purpose of this study was to characterize the prevalence and types of plasmid replicons carried by 205 *Salmonella* isolates isolated from cattle, and to examine the transmissibility of these plasmids by conjugation.

Materials and Methods

Salmonella *isolates*

S. enterica serotype Newport isolates designated SN1-SN50 and SN52-SN100 were obtained from the U.S. Meat Animal Research Center, Agricultural Research Service, U.S. Department of Agriculture (USDA), Clay Center, NE. These isolates were obtained from preevisceration carcasses of cull cows, bulls, and fed cattle, collected at four geographically distinct processing facilities in the northwest, northeast, southeast, and mid-western United States. Of the 99 *Salmonella* Newport isolated at slaughter, 89% were from cull cattle, including cows, bulls, and dairy cattle, while 11% were from fed beef cattle.

One hundred and six additional *S. enterica* isolates were collected from fecal grab samples of dairy cattle on farm in Texas and New Mexico and have been epidemiologically described elsewhere (Bischoff *et al.*, 2004; Edrington *et al.*, 2004a, 2004b). These isolates were designated using initials to identify the corresponding serovar and isolate number (101–205), and included *Salmonella* Newport (SN101–119, SN121–123, SN150–152, and SN205–SN206); *Salmonella* Reading (SR161–166, SR174–192, SR194–196, SR198–200, and SR202–204); *Salmonella* Kinshasa (SK120, SK125, SK130–133, SK135–146, and SK148–149); *Salmonella* Typhimurium (ST167–172, ST193, ST197, and ST201); *Salmonella* Give (SG153–160); *Salmonella* Agona (SA127-129, SA134, SA147, SA173); and *Salmonella* Infantis (SI124 and SI126).

Determination of antimicrobial susceptibility

The antimicrobial minimum inhibitory concentrations (MICs) were determined by broth microdilution according to the Clinical Laboratory Standards Institute (CLSI) (CLSI, 2003). Susceptibility testing was performed with a Sensititre® automated antimicrobial susceptibility system according to the manufacturer's instructions (Trek Diagnostic Systems, Westlake, OH). NARMS panels (CMV1AGNF) for Enterobacteriaceae were used in the Sensititre system; the following antimicrobials were assayed: amikacin, amoxicillin/clavulanic acid, ampicillin, cefoxitin, ceftiofur, ceftriaxone, chloramphenicol, ciprofloxacin, gentamicin, kanamycin, nalidixic acid, streptomycin, sulfamethoxazole, tetracycline, and trimethoprim/sulfamethoxazole. The following ATCC strains were used as controls for antimicrobial susceptibility testing: *E. coli* 25922, *Enterococcus faecalis* 29212, *Staphylococcus aureus* 29213, and *Pseudomonas aeruginosa* 27853. Data were interpreted using CLSI breakpoints unless unavailable, and then breakpoints from the NARMS 2004 annual report were used (CLSI, 2005; FDA, 2004). Because rifampicin was not on the CMV1AGNF plate, its MIC was determined manually by broth microdilution using the methods described by the CLSI (CLSI, 2003).

PBRT

PCR replicon typing of *Salmonella* isolates and transconjugant *E. coli* isolates was performed using the method of Carattoli *et al.* (2005). The replicon types tested included B/O, K, $FIIA_s$, FIA, FIB, FIC, HI1, HI2, Y, I1, repF, X, L/M, N, P, W, T, and A/C. Positive controls for these replicons were provided by Istituto Superiore di Sanità, Rome, Italy.

Molecular analysis

Total genomic DNA was prepared according to manufacturer's instructions using the DNeasy Tissue Kit (Qiagen, Valencia, CA). The primers used to amplify bla_{CMY} gene were forward 5′-ATAACCACCCAGTCACGC-3′ and reverse 5′-CAGTAGCGAGACTGCGCA-5′ (Rankin *et al.*, 2002). PCR primer sequences used to map the transfer gene regions of A/C replicons are given in (Table 1), and their positions on the plasmid relative to the *tra* genes are shown in (Fig. 1). EMBL GenBank accession numbers used for comparisons and primer design were CP000602 and CP000604 (Welch *et al.*, 2007). Alignments were made using the BLAST program available at the National Center for Biotechnology Information (Altschul *et al.*, 1997). Primers were designed based on the region of interest. JM109 and *Salmonella* Newport isolate SN50 were used as negative controls.

PCR reactions (50 μL) contained template DNA (100 ng), 25 pmol of each of the selected primers, 25 μL of HotStarTaq Mastermix (Qiagen), and water to the required volume. Amplification for mapping was as follows: tubes were heated to 95°C for 15 min, followed by 35 cycles of PCR with a denaturation temperature of 94°C (1 min), and annealing temperature of 58°C (1 min), and an extension temperature of 72°C (2 min).

Amplicons for sequencing were purified by the Qiaquick PCR Purification Kit (Qiagen) and submitted to the DNA Core Facility in the Department of Veterinary Pathobiology, Texas A&M University (College Station, TX). Sequence comparisons

Table 1. Primers Corresponding to A/C Plasmid in *Yersinia ruckeri* and *Salmonella* Newport

Primer name	*Sequence 5′-3′*	Yersinia ruckeri	Salmonella *Newport*
CmyFW2	CGGCCTGGCGCATTCTTGAAAAGC	Not present	73481–73505, 68825–68848
HypRV1	CGAAATCATGTTGGCTCATGATCCC	64820–64840	77471–77492, 68825–68848
HypFW2	CTCTGGTTGGGGTCGTGACT	67961–67981	80512–80632, 61697–61717
TraAFW2	GGCTGTTGTGCAACTCAGCAATG	62248–62270	58828–58850
TraCRV1	GTGCTGCGGGATCAACGTTTC	68899–68919	81540–81570

EMBL GenBank accession numbers used for comparisons were CP000602 (*Y. ruckeri*) and CP000604 (*Salmonella* Newport).

to the A/C replicon *repA* gene (EMBL GenBank accession no. X73674) were made using the BLAST program available at the National Center for Biotechnology Information (Altschul *et al.*, 1997).

Bacterial conjugation

Conjugation experiments using *Salmonella* donor isolates were done on solid support, 0.45 μm, 13 mm filters (Millipore, Billerica, MA) with nalidixic acid– resistant and rifampicin-resistant strains JM109 *F′* (provided by Dr. Kenneth Bischoff), *E. coli* DH5α F^- (Invitrogen, Carlsbad, CA), enterohemorrhagic *E. coli* CVM1572, and pansusceptible *Salmonella* Newport SN50 as the recipient strains. Enterohemorrhagic *E. coli* CVM1548 (donor control) and CVM1572 have been previously described in filter conjugations (Bischoff *et al.*, 2005; Poole *et al.*, 2006). To make the recipient strains resistant to nalidixic acid, each was grown over night at 37°C in brain heart infusion broth (BHIB) supplemented with 4.0, 8.0, 16.0, and 32.0 μg/mL nalidixic acid. One hundred microliters from a turbid broth culture with the highest concentration of nalidixic acid was inoculated into BHIB with the next highest concentration of nalidixic acid until the culture grew at a nalidixic acid concentration of 32.0 μg/mL. This procedure was repeated with rifampicin. The resistant strain was then streaked for isolation on brain heart infusion agar with 32.0 μg/mL of nalidixic acid and rifampicin and grown overnight at 37.0°C to confirm its resistance to both antimicrobials. JM109 was positive for FIA, FIB, and FIC by PBRT. *E. coli* DH5α, CVM1572, and SN50 were negative for all replicons tested by PBRT.

Conjugations using JM109 as the recipient were done in triplicate. These conjugations were done at 37°C and 30°C. Conjugations using DH5α as the recipient were done once at 37°C unless the donor isolates possessed an H replicon, and then conjugations were done at 30°C. JM109 was chosen as a recipient because it had a high conjugation frequency when used in conjugations with *Salmonella* isolates (Bischoff *et al.*, 2004b) and because transconjugants could be validated using PBRT for FIA, FIB, and FIC. DH5α was chosen because it is *F′* minus. For each replicate, the *Salmonella* isolates and *E. coli* CVM1548 were grown overnight at the specified temperature in BHIB (Difco, Detroit, MI) with a single antimicrobial agent (32 μg/mL tetracycline, 8 μg/mL ceftiofur, or 32 μg/mL chloramphenicol). Each *E. coli* recipient was grown overnight at the specified temperature in BHIB with 32 μg/mL nalidixic acid and rifampicin.

Tetracycline was chosen as the primary counter selection agent because it could be used for all of the resistant isolates, and it is an antimicrobial agent frequently used in cattle production systems. Counter selection with 8.0 μg/mL ceftiofur or 32 μg/mL chloramphenicol, in place of tetracycline,

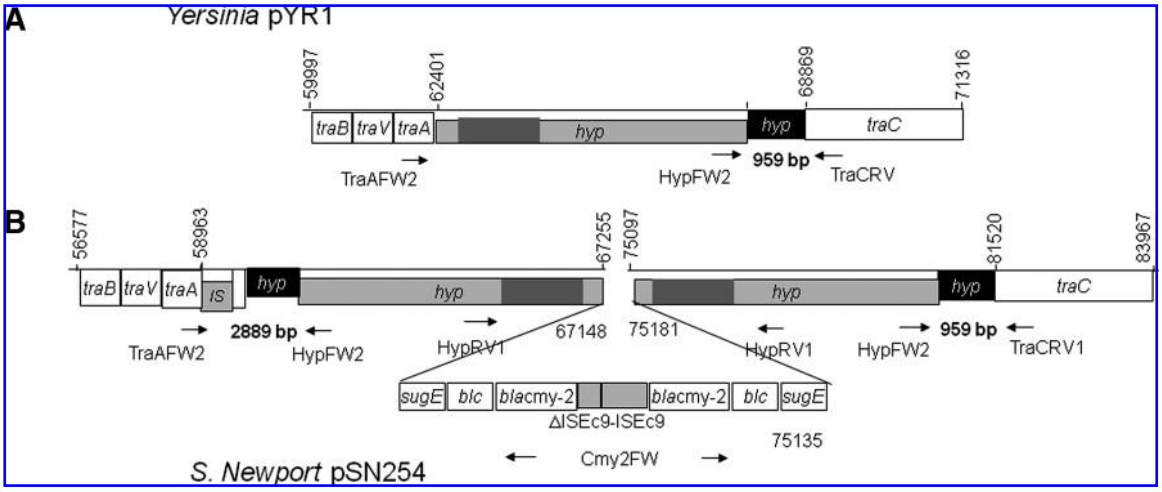

FIG. 1. Partial physical and genetic structure of the incompatibility A/C plasmid replicon transfer gene regions (not to scale) showing the positions of the primers used to map the bla_{CMY} insertion. (**A**) Map of *Yersinia ruckeri* pYR1 that does not contain a bla_{CMY} insertion (EMBL GenBank CP000602). (**B**) Map of *Salmonella* Newport pSN254 that contains bla_{CMY} insertion and inverted duplication of a hypothetical protein gene (EMBL GenBank CP000604).

was also done for appropriate isolates. Putative transconjugant colonies were confirmed as *E. coli* using an indole spot test and four indole-positive transconjugant colonies were subcultured to blood agar for susceptibility testing and molecular analysis.

Pulsed-field gel electrophoresis analysis

Pulsed-field gel electrophoresis (PFGE) analysis was performed according to the protocol developed by the Centers for Disease Control and Prevention (Ribot *et al.*, 2006). Agarose-embedded DNA was digested with *Xba*I (New England Biolabs, Beverly, MA). *Salmonella* serotype Braenderup strain H9812 was used as a control and for standardization of gels. Banding patterns were analyzed and compared using Bionumerics software (Applied Maths, Sint-Martens-Latem, Belgium), employing the Dice similarity coefficient with a 1.5% band position tolerance, in conjunction with the unweighted pair group method, using arithmetic averages for clustering.

Results

Susceptibility testing and plasmid replicon typing of Salmonella *isolates*

The results of susceptibility testing and replicon typing done on 205 *Salmonella* isolates are shown in (Table 2). Two of the cull cattle and all of the fed cattle isolates were pansusceptible. Eighty-six percent ($n = 177$) of the 205 isolates studied were resistant to multiple (3–12) antimicrobial agents. Eighty-two percent ($n = 146$) of the MDR isolates ($n = 177$) were resistant to nine or more antimicrobial agents, displayed resistance to extended-spectrum cephalosporins, and carried the A/C replicon with bla_{CMY} (Table 2).

A/C was the predominant replicon detected in 90% ($n = 160$) of the MDR isolates. Fourteen *Salmonella* Newport isolates that displayed resistance to four to five antimicrobials carried the A/C replicon, but lacked bla_{CMY}; seven of these possessed only A/C, and seven were positive for a second replicon. Overall, multiple replicons were detected in 25% ($n = 45$) of the MDR isolates (Table 2). Thirty-seven isolates carried coresident replicons with A/C; these included I1, N, B/O, HI1, and HI2 (Table 2). All of the *Salmonella* isolates were susceptible to ciprofloxacin and nalidixic acid, and displayed MICs against rifampicin of $\leq 8\,\mu g/mL$.

Conjugative transfer of plasmids

Twenty-six percent of the *Salmonella* isolates ($n = 54$) produced transconjugants when tetracycline was used as the counter selection agent for plasmid transfer (Table 3). All of the isolates capable of transferring bla_{CMY} when tetracycline was used for counter selection did so when ceftiofur was substituted ($n = 27$). *Salmonella* isolates that were positive by PBRT for the coresident replicons A/C-I1, A/C-HI2, A/C-N, and A/C-B/O transferred their genotypes and phenotypes to all of the recipients tested (Table 3). Four MDR-AmpC *Salmonella* Newport donors, SN2, SN21, SN27, and SN82, possessed A/C bla_{CMY}-positive plasmids, but no other detectable plasmids. These four isolates produced two types of transconjugants those positive for the A/C bla_{CMY}-positive plasmid and those negative for A/C bla_{CMY}-positive plasmid. The transconjugants that were negative for A/C bla_{CMY} did not display resistance to extended-spectrum cephalosporins. This suggested the presence of undetectable coresident plasmids in the donor strains that carried tetracycline resistance.

The 14 *Salmonella* Newport isolates that possessed A/C bla_{CMY}-negative plasmids produced transconjugants when tetracycline or chloramphenicol was used for counter selection. This included two that carried I1 and five that carried HI2; the latter five required conjugation at 30°C.

Conjugation frequency of selected Salmonella *Newport isolates using different recipients*

All isolates that produced transconjugants when JM109 was used as the recipient ($n = 54$) also produced transconjugants when DH5α was used as the recipient. Six *Salmonella* Newport isolates were selected for examination of conjugation frequency to one *Salmonella* Newport and three *E. coli* recipients (Table 4). The results of these conjugation studies indicated that wild-type strain *E. coli* CVM1572 and *E. coli* JM109 had higher conjugation frequencies than *E. coli* DH5α (F minus) or *Salmonella* Newport SN50. SN11 transferred the N replicon to JM109, but not the A/C replicon. However, when CVM1572 was used as the recipient, both N and A/C were transferred. N was detected in all of the transconjugants, and A/C was detected in 50% of the transconjugants. The seven *Salmonella* Newport isolates that possessed only the A/C bla_{CMY}-negative plasmids produced transconjugants in JM109, DH5α, and CVM1572, but not in the *Salmonella* Newport recipient.

PFGE of 99 Salmonella *Newport isolates*

PFGE was done on the 99 *Salmonella* Newport isolates collected at slaughter; selected isolates are shown in Fig. 1. The MDR isolates segregated into two PFGE types (1 and 2) with 84.9% similarity. The pansusceptible isolates composed PFGE type 3. The four conjugative isolates that may have possessed undetectable plasmids, SN2, SN21, SN27, and SN82, are labeled with asterisks (Fig. 2). Three of these were PFGE type 1 isolates, and the fourth is PFGE type 2. SN21 has 100% similarity with the *Salmonella* Newport isolates that were conjugative and possessed A/C bla_{CMY}-negative plasmids. In this same clade SN39 possessed the IncI as a coresident plasmid. The isolates within this clade as well as SN2, SN27, and SN82 all show differences from the isolates that possess nontransmissible A/C plasmids.

PCR mapping of the transfer gene region of the A/C plasmids with and without bla_{CMY} *from* Salmonella *Newport isolates*

PCR mapping of 10 A/C bla_{CMY}-positive *Salmonella* Newport isolates ($n = 6$, PFGE type 1; $n = 4$, PFGE type 2) indicated that the bla_{CMY} gene was present as an inverted repeat element on the A/C plasmid in between the *traA* and *traC* genes (Fig. 1). PCR of these 10 isolates produced the expected 2889 bp amplicon when the *traA*-inverted *hyp* gene junction region was amplified. This demonstrated the presence of the inverted *hyp* gene adjacent to the *traA* gene. No PCR amplicons representing this junction were detectable for the seven A/C bla_{CMY}-negative *Salmonella* Newport isolates (PFGE type 2). PCR of the 10 *Salmonella* Newport that possessed A/C bla_{CMY}-positive plasmids produced the expected 959 bp band when the *traC–hyp* gene junction was amplified. Amplifica-

Table 2. The Number of *Salmonella* Isolates ($N = 205$) Categorized by Serovar, Resistance Profile, and Replicon Genotype

	Newport (n = *126*)	*Reading* (n = *34*)	*Kinshasa* (n = *20*)	*Agona* (n = *6*)	*Infantis* (n = *2*)	*Give* (n = *8*)	*Typhimurium* (n = *9*)	
Resistance phenotype	*No. of isolates, replicon(s)*	*No. of isolates, replicon(s)*	*No. of isolates, replicon(s)*	*No. of isolates, replicon(s)*	*No. of isolates, replicon(s)*	*No. of isolates, replicon(s)*	*No. of isolates, replicon(s)*	*Presence of* bla_{CMY}
AkAmApFT(Ax)CGKSSuTe	3 A/C		3 A/C, B/O					+
AmApFT(Ax)CGKSSuTe	1 A/C, B/O	30 A/C	17 A/C, B/O	5 A/C, HI1	2 A/C, B/O			+
AmApFT(Ax)CKSSuTe	23 A/C			1 A/C				+
AmApFTAxCKSSuTe	27 A/C							+
AmApFT(Ax)CGSSuTe		3 A/C						+
AmApFT(Ax)CSSuTe	16 A/C							+
AmApFTAxCSSuTe	13 A/C							+
AmApFT(Ax)CSSuTe	2 A/C, N							+
ApCSSuTe							0	−
CKSSuTe	3 A/C							−
CKSSuTe	5 A/C, HI2							−
CSSuTe	4 A/C							−
CSSuTe	2 A/C, I1							−
KSTe						8 FIA, FIB, I1		−
Pansusceptible	24 None	1 None						−
Pansusceptible	1 I1							−
Pansusceptible	1 A/C							−
Pansusceptible	1 FIC							−

Resistance phenotype is defined as resistance to the following antimicrobial agents: Ak, amikacin; Am, amoxicillin/clavulanic acid; Ap, ampicillin; F, cefoxitin; T, ceftiofur; Ax, ceftriaxone (Ax), intermediate susceptibility to ceftriaxone (16–32 mg/L); C, chloramphenicol; Cp, ciprofloxacin; G, gentamicin; K, kanamycin; N, nalidixic acid; R, rifampicin; S, streptomycin; Su, sulfizoxazole; Te, tetracycline; Sxt, trimethoprim/sulfamethoxazole. Isolates resistant to extended-spectrum cephalosporins carried the bla_{CMY} gene.

"+" symbol indicates that all isolates with this phenotype were positive for bla_{CMY2}.

Table 3. Transconjugant Phenotypes and Genotypes Obtained from *Salmonella* Donors with the JM109 Recipient

Donor replicon type	*Donor resistance phenotype*	bla_{CMY}	*Donor*	*Transconjugant resistance phenotype*	*Transconjugantreplicon type*	*Transconjugant* bla_{CMY}
A/C, B/O	AmApFT(Ax)CGKSSuTe	+	*Salmonella* Newport, $n=1$	AmApFT(Ax)CGKSSuTeNR	A/C, B/O	+
A/C, B/O	AmApFT(Ax)CGKSSuTe	+	*Salmonella* Infantis, $n=2$	AmApFT(Ax)CGKSSuTeNR	A/C, B/O	+
A/C, B/O	AmApFT(Ax)CGKSSuTe	+	*Salmonella* Kinshasha, $n=16$	AmApFT(Ax)CGKSSuTeNR	A/C, B/O	+
A/C, B/O	AkAmApFT(Ax)CGKSSuTe	+	*Salmonella* Kinshasha, $n=3$	AkAmApFT(Ax)CGKSSuTeNR	A/C, B/O	+
A/C, I1	CSSuTe	−	*Salmonella* Newport, $n=2$	CSSuTeNR	A/C, I1	−
A/C, N	AmApFTAxCSSuTe	+	*Salmonella* Newport, $n=1$ (SN11)	AmCSSuTeNR	N	−
A/C, N	AmApFTAxCSSuTe	+	*Salmonella* Newport, $n=1$ (SN14)	AmApFTAxCSSuTeNR	A/C, N	+
A/C	AmApFTAxCKSSuTe	+	*Salmonella* Newport, $n=2$	AmApFTAxCKSSuTeNR; CKSSuTeNR	A/C, untypable	+ −
A/C	AmApFT(Ax)CSSuTe	+	*Salmonella* Newport, $n=2$	AmApFT(Ax)CSSuTeNR; CSSuTeNR	A/C, untypable	+ −
A/C	CKSSuTe	−	*Salmonella* Newport, $n=3$	CKSSuTeNR	A/C	−
A/C	CSSuTe	−	*Salmonella* Newport, $n=4$	CSSuTeNR	A/C	−
FIA, FIB, I1	KSTe	−	*Salmonella* Give, $n=8$	KSTeNR	FIA, FIB, I1; FIA, FIB	− −
A/C, HI2	CKSSuTe	−	*Salmonella* Newport, $n=5$	CKSSuTeNR	A/C, HI2	−
A/C, HI1	AmApFT(Ax)CGKSSuTe	−	*Salmonella* Agona, $n=4$	GKSTeNR	HI1	−

Resistance phenotype is defined as resistance to the following antimicrobial agents: Ak, amikacin; Am, amoxicillin/clavulanic acid; Ap, ampicillin; F, cefoxitin; T, ceftiofur; Ax, ceftriaxone (Ax), intermediate susceptibility to ceftriaxone (16–32 mg/L); C, chloramphenicol; Cp, ciprofloxacin; G, gentamicin; K, kanamycin; N, nalidixic acid; R, rifampicin; S, streptomycin; Su, sulfizoxazole; Te, tetracycline; Sxt, trimethoprim/sulfamethoxazole.

TABLE 4. CONJUGATION FREQUENCIES WITH *ESCHERICHIA COLI* AND *SALMONELLA* NEWPORT RECIPIENTS

		Recipient		*Strains*	
		Escherichia coli *JM109*	E. coli *CVM1572*	Salmonella *Newport SN50*	E. coli *DH5α*
Donor strain	*Inc type*	*Conjugation frequency*			
CVM1548	I1, FIB, FIC, P	$6.37\times10^{-02}\pm1.2\times10^{-02}$	$2.01\times10^{-01}\pm7.4\times10^{-02}$	$5.19\times10^{-07}\pm1.9\times10^{-07}$	$6.70\times10^{-03}\pm2.4\times10^{-03}$
SN4	A/C	NTD	NTD	NTD	NTD
SN11	A/C, N	$5.65\times10^{-02}\pm2.8\times10^{-02}$	$1.05\times10^{-01}\pm3.7\times10^{-02}$	$6.46\times10^{-03}\pm6.29\times10^{-03}$	$4.53\times10^{-03}\pm2.3\times10^{-03}$
SN14	A/C, N	$1.40\times10^{-02}\pm3.6\times10^{-03}$	$4.42\times10^{-02}\pm4.4\times10^{-02}$	$5.74\times10^{-04}\pm7.03\times10^{-05}$	$7.30\times10^{-03}\pm2.4\times10^{-03}$
SN39	A/C, I1	$3.60\times10^{-03}\pm1.2\times10^{-03}$	$6.94\times10^{-02}\pm4.6\times10^{-02}$	$3.12\times10^{-05}\pm4.1\times10^{-05}$	$5.23\times10^{-04}\pm4.9\times10^{-04}$
SN40	A/C, I1	$6.57\times10^{-04}\pm8.4\times10^{-03}$	$6.12\times10^{-02}\pm8.1\times10^{-02}$	$2.93\times10^{-07}\pm1.5\times10^{-07}$	$8.14\times10^{-04}\pm8.5\times10^{-05}$
SN82	A/C	$7.12\times10^{-02}\pm8.1\times10^{-03}$	$5.37\times10^{-02}\pm4.1\times10^{-02}$	$2.02\times10^{-07}\pm2.8\times10^{-07}$	$9.78\times10^{-03}\pm1.3\times10^{-03}$

Conjugation frequency is defined by the number of transconjugants divided by the number of recipient cells. The mean and standard deviation for two independent conjugations are shown.
NTD, no transconjugants detected; Inc, incompatibility.

tion of the bla_{CMY}–*hyp* gene junction (4011 bp) confirmed that bla_{CMY} was adjacent to the A/C *hyp* gene in the 10 isolates that possessed A/C bla_{CMY}-positive plasmids.

Sequence analysis of the A/C repA *gene*

Because A/C was the most prevalent replicon detected among the isolates characterized in this study it was of interest to determine if it was the A/C_2 variant that has been identified in a few *Salmonella* and *E. coli* isolates from the United States. Therefore, the 890 bp A/C *repA* gene was sequenced from one *Salmonella* Newport isolate, SN4. The resulting sequence was compared and found to be identical to the A/C_2 variant described from plasmids that carried extended-spectrum cephalosporin resistance and had been isolated in the United States.

Discussion

Food animal producers are interested in farm management practices that will prevent infection and future dissemination of MDR pathogens. To better understand how *Salmonella* acquires and disseminates antimicrobial resistance, this project characterized the prevalence and transmissibility of resistance plasmids present in *Salmonella* isolated from bovine sources. As expected, the A/C replicon was the predominant replicon in cephalosporin-resistant *Salmonella* (Carattoli *et al.*, 2006). B/O was the second most prevalent replicon detected at 13%. B/O was only found in combination with A/C from on farm dairy cattle isolates. Two HI replicons (HI1 and HI2) that are known to be temperature sensitive for conjugation (Sherburne *et al.*, 2000) were also present in *Salmonella* Newport and *Salmonella* Agona isolates from on farm dairy cattle.

An important observation from the conjugation studies was that A/C plasmids encoding the MDR-AmpC resistance phenotype rarely transferred A/C when it was the only replicon detected in the donor isolate. Approximately 9% ($n = 11$) of the MDR *Salmonella* isolates that carried A/C alone were able to produce *E. coli* transconjugants when either tetracycline or ceftiofur were used for counter selection.

Transconjugants produced from MDR-AmpC *Salmonella* isolates that were positive for the coresident replicon pairs, B/O-A/C and I1-A/C, always received both replicons. The consistent cotransfer suggests that both plasmids may have been required for successful conjugation and counter selection with tetracycline and ceftiofur. The B/O and I1 replicons may have been necessary for transfer, whereas A/C may have been necessary for resistance to tetracycline and/or ceftiofur. It is also possible that both replicons were present on the same plasmid (Couturier *et al.*, 1988). Isolation, purification, and sequencing of some of the plasmids detected in this study are underway. Both HI2 and A/C always transferred at 30°C when tetracycline or chloramphenicol was used for counter selection. Since it is known that HI plasmids are temperature sensitive for conjugation, this suggested conjugation was dependent on HI2 in the five strains that carried HI2 and A/C (Sherburne *et al.*, 2000). A/C did not transfer with HI1 under the conditions used in this study.

In studies of conjugation frequency using five different *Salmonella* Newport donors with three different *E. coli* recipients and one *Salmonella* Newport recipient, the *Salmonella* Newport recipient demonstrated the lowest conjugation frequency. The enterohemorrhagic *E. coli* CVM1572 isolate

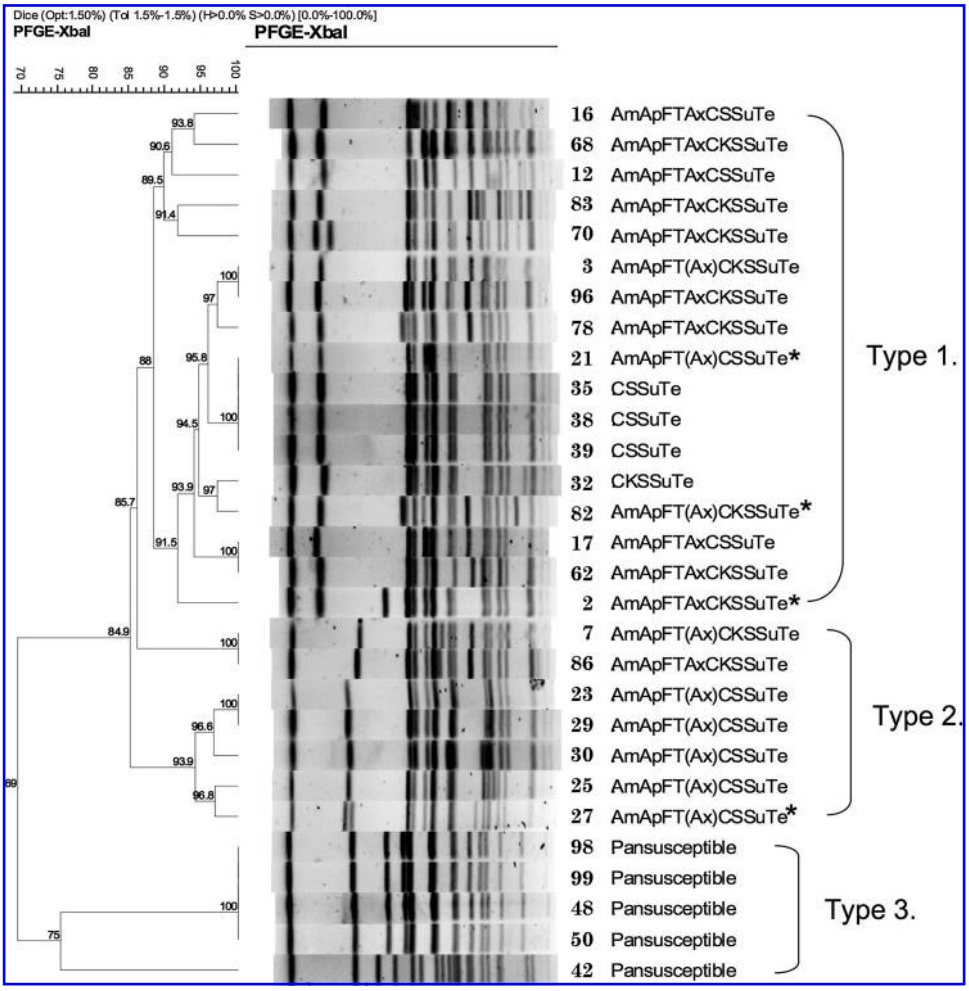

FIG. 2. Dendrogram with percent similarity of 29 representative *Salmonella* Newport isolates obtained by cluster analysis of 99 *Salmonella* Newport hide isolates. The pulsed-field gel electrophoresis (PFGE) banding pattern is followed by the *Salmonella* Newport (SN) isolate number, antimicrobial resistance phenotype, and PFGE type.

demonstrated the highest conjugation frequency. CVM1572 was originally isolated from a swine farm in Oklahoma that was experiencing a diarrhea outbreak. Whether the virulent nature of this isolate contributed to its ability to be a suitable recipient for incompatibility plasmid transfer is unknown.

Possibly, the most interesting observation was that all seven of the MDR *Salmonella* Newport strains that carried only A/C bla_{CMY}-negative plasmids were conjugative with three *E. coli* recipients, but not the *Salmonella* Newport recipient. The conjugation frequency may have been too low for detection when SN50 was used as the recipient. These seven donor isolates were also conjugative when tetracycline or chloramphenicol was used for counter selection.

Recently, the complete sequence of the A/C plasmid replicon (pSN254) associated with *S. enterica* Newport was published (Welch *et al.*, 2007). This showed the presence of a bla_{CMY}-*hyp* inverted repeat element integrated into the *tra* gene operon on the A/C plasmid backbone. The *tra* genes encode proteins that are believed to be necessary for conjugative transfer. PCR mapping of the *traA* and *traC* regions of the A/C bla_{CMY}-positive plasmid in 10 *Salmonella* Newport isolates from two PFGE types revealed the same bla_{CMY} inverted repeat element. Attempts at mapping the bla_{CMY} region using primers designed from GenBank accession DQ164214 deposited by (Kang *et al.*, 2006) were unsuccessful. Mapping of a much larger number of A/C replicons from other *Salmonella* and Enterobacteriaceae is necessary to determine if the A/C plasmid backbone containing the inverted repeat element is the most prominent in the United States. However, sequence analysis of the A/C *repA* gene from one isolate that possessed the bla_{CMY}-*hyp* inverted repeat element was identical to the A/C_2 *repA* gene described as a U.S. variant (Carattoli *et al.*, 2006).

The bla_{CMY}-*hyp* inverted repeat element was lacking in those *Salmonella* Newport isolates that possessed A/C bla_{CMY}-negative plasmids. The combined mapping and conjugation data presented here suggest that A/C may have been a conjugative plasmid before the integration of bla_{CMY} into the *tra* operon. Integration of bla_{CMY} may have reduced conjugative activity of A/C bla_{CMY}-positive plasmids to undetectable levels. Additional studies to inactivate the transfer region of A/C bla_{CMY}-negative plasmids are planned to determine if this region is essential for conjugative transfer of A/C. The A/C bla_{CMY}-negative plasmids in this collection may repre-

sent an early ancestor to the A/C bla_{CMY}-positive plasmid that confers resistance to cephalosporins. Comparative sequence analysis of multiple A/C plasmid backbone from *Salmonella* and *E. coli* will also aid in elucidating the evolution and epidemiologic dissemination of the A/C plasmid.

The data collected during this study also suggested that coresident conjugative plasmids may play a role in the conjugative transfer of A/C bla_{CMY}-positive plasmids from *Salmonella* isolates. More extensive mobilization studies *in vitro* and *in vivo* are necessary to determine the validity of this hypothesis. Whether this has practical implications with regard to dissemination of A/C in *Salmonella* isolates in food-animal production facilities across the United States is unknown.

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Disclaimer

Proprietary or brand names are necessary to report factually on available data; however, the USDA neither guarantees nor warrants the standard of the product, and the use of the name by the USDA implies no approval of the product, or exclusion of others that may be suitable.

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